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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 47/48 A1

(11) International Publication Number: WO 94/17831

(43) International Publication Date: 18 August 1994 (18.08.94)

(21) International Application Number: PCT/IB94/00011

(22) International Filing Date: 8 February 1994 (08.02.94)

(30) Priority Data:
08/016,354 11 February 1993 (11.02.93) US

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(81) Designated States: AU, CA, CN, CZ, FI, HU, JP, KR, NO, NZ, PL, RU, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A COMBINATION OF NEUROTROPHIN AND ANTIBODY DIRECTED TOWARD MYELIN-ASSOCIATED NEURITE GROWTH INHIBITORY PROTEIN PROMOTES CENTRAL NERVOUS SYSTEM REGENERATION

(57) Abstract

The present invention relates to methods of promoting central nervous system regeneration in a subject in need of such treatment comprising administering a therapeutically effective amount of an essentially purified and isolated neurotrophin family member together with an antibody directed toward a myelin-associated neurite growth inhibitory protein.

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A COMBINATION OF NEUROTROPHIN AND ANTIBODY DIRECTED TOWARD MYELIN-ASSOCIATED NEURITE GROWTH INHIBITORY PROTEIN PROMOTES CENTRAL NERVOUS SYSTEM REGENERATION

1. INTRODUCTION

The present invention relates to methods of promoting central nervous system regeneration in a subject in need of such treatment comprising administering a therapeutically effective amount of an essentially purified and isolated neurotrophin family member together with an antibody directed toward a myelin-associated neurite growth inhibitory protein.

2. BACKGROUND OF THE INVENTION

Cell attachment, cell spreading, cell motility, and, in particular, neurite outgrowth are strongly dependent on cell-substrate interactions (Sanes, 1983, Ann. Rev. Physiol. 45:581-600; Carbonetto et al., 1987, J. Neurosci. 7:610-620). An increasing number of substrate molecules favoring neuroblast migration or neurite outgrowth have been found in central and peripheral nervous tissue (Cornbrooks et al., 1983, Proc. Natl. Acad. Sci. USA 80:3850-3854; Edelman, 1984, Exp. Cell Res. 161:1-16; Liesi, 1985, EMBO J. 4:1163-1170; Chiu, A.Y. et al., 1986, J. Cell Biol. 103:1383-1398; Fischer et al., 1986, J. Neurosci. 6:605-612; Lindner et al., 1986, Brain Res. 377:298-304; Mirsky et al., 1986, J. Neurocytol. 15:799-815; Stallcup et al., 1986, J. Neurosci. 5:1090-1101; Carbonetto et al., 1987, J. Neurosci. 7:610-620). The appearance of some of these factors can be correlated with specific developmental stages, and, in the peripheral nervous system (PNS), also with denervation (Edelman, 1984, Exp. Cell Res. 161:1-16; Liesi, 1985, EMBO J. 4:1163-1170; Stallcup et al., 1985, J. Neurosci. 5:109-1101; Daniloff et al., 1986, J. Cell

Biol. 103:929-945; Carbonetto et al., 1987, J. Neurosci. 7:610-620).

However, the differentiated central nervous system (CNS) of higher vertebrates is capable of only very limited regenerative neurite growth after lesions. Limited regeneration after lesion has been seen in the retina (McConnell and Berry, 1982, Brain Res. 241:362-365) and in aminergic unmyelinated fiber tracts after chemical (Bjorklund and Stenevi, 1979, Physiol. Rev. 59:62-95) but not mechanical lesions (Bregman, 1987, Dev. Brain Res. 34:265-279). Neurite growth from implanted embryonic CNS tissues in adult rat CNS has been found in some cases to reach up to 14 mm within some gray matter areas, but has not been found to exceed 1 mm within white matter (Nornes et al., 1983, Cell Tissue Res. 230:15-35; Bjorklund and Stenevi, 1979, Physiol. Rev. 59:62-95; Commission, 1984, Neuroscience 12:839-853). On the other hand, extensive regenerative growth has been found in the CNS of lower vertebrates and in the peripheral nervous system of all vertebrates including man.

Results from transplantation experiments indicate that the lack of regeneration is not an intrinsic property of CNS neurons, as these readily extend processes into implanted peripheral nervous tissue (Benfey and Aguayo, 1982, Nature (London) 296:150-152; Richardson et al., 1984, J. Neurocytol. 13:165-182 and So and Aguayo, 1985, Brain Res. 328:349-354). PNS neurons, however, failed to extend processes into CNS tissue, thus indicating the existence of fundamental differences between the two tissues (Aguayo et al., 1978, Neurosci. Lett. 9:97-104; Weinberg and Spencer, 1979, Brain Res. 162:273-279).

One major difference between PNS and CNS tissue is the differential distribution of the neurite

outgrowth promoting extracellular matrix component laminin (Liesi, 1985, EMBO J. 4:2505-2511; Carbonetto et al., 1987, J. Neurosci. 7:610-620), but other factors may be involved. Drastic differences have been observed in neurite growth supporting properties of sciatic and optic nerve explants in vitro, in spite of the presence of laminin immunoreactivity in both explants (Schwab and Thoenen, 1985, J. Neurosci. 5:2415-2423).

It has been suggested that the differentiated CNS may lack cellular or substrate constituents that are conducive for neurite growth during development (Liesi, 1985, EMBO J. 4:2505-2511; and Carbonetto et al, 1987, J. Neurosci. 7:610-620), or it may contain components which are nonpermissive or inhibitory for nerve fiber regeneration (Schwab and Thoenen, 1985, J. Neurosci. 5:2415-2423).

Recently, a growth (cell proliferation) inhibitory factor for mouse neuroblastoma cells was partially purified and characterized from the culture medium of fetal rat glioblasts as well as from C6 rat glioma cells (Sakazaki et al., 1983, Brain Res. 262:125-135). The factor was estimated to have a molecular weight of about 75,000 by gel filtration with BioGel P-20 with an isoelectric point of 5.8. The factor did not appear to alter the growth rate or morphology of glial cells (C6) or fibroblasts (3T3). In addition, no significant nerve growth inhibitory factor activity was detected towards neuroblastoma cells (Neuro La, NS-20Y and NIE-115) or cloned fibroblasts (3T3).

CNS myelin-associated proteins have been identified that inhibit neurite outgrowth. Two oligodendrocyte-and myelin-associated membrane proteins; NI-35 (35kD) and NI-250 (250kD), with potent

inhibitory effects on neurite growth, were identified by in vitro and biochemical studies (Schwab and Caroni, 1988, J. Neurosci. 8:2381-2393; Caroni and Schwab, 1988, J. Cell. Biol. 106:1281-1288). Monoclonal antibody IN-1, which neutralizes the activity of these constituents in various systems, has been shown to lead to regeneration of corticospinal tract (CST) axons in young rats over distances of up to 5-11 mm distal to a spinal cord lesion within 2 weeks (Int. Application No. 89912786.4, filed November 2, 1989, by Schwab et al.; U. S. Serial No. 07/401,212 by Schwab et al., filed August 30, 1989; U. S. Serial No. 07/719,692 by Schwab et al., filed June 24, 1991). Such antibody has been used to demonstrate the role of myelin-associated neurite growth inhibitors in the absence of regeneration of lesioned CNS fiber tracts observed under normal conditions.

3. SUMMARY OF THE INVENTION

The present invention relates to methods of promoting central nervous system regeneration in a subject in need of such treatment comprising administering a therapeutically effective amount of an essentially purified and isolated neurotrophin family member together with an antibody directed toward a myelin-associated neurite growth inhibitory protein. It is based, at least in part, on the discovery that monoclonal antibody IN-1, directed against a myelin-associated neurite growth inhibitor, together with a member of the neurotrophin family, (e.g. neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), or nerve growth factor (NGF)), was able to promote regeneration of neurites over long distances in the partially transected spinal cord of adult rats. Such distances significantly exceeded the regeneration

resulting from antibody without neurotrophin family member.

In preferred embodiments of the invention, NT-3, together with antibody directed toward myelin-associated neurite growth inhibitor, may be used to promote regeneration in the CNS. Such methods may be directed toward the treatment of neurologic disorders, including trauma as well as degenerative conditions.

4. DESCRIPTION OF THE FIGURES

FIGURE 1. Sprouting of lesioned corticospinal tract fibers. The sprouting index was calculated by subtracting the branching index of normal, unlesioned animals. A. Sprouting at the lesion site, 1mm rostral to lesion, and 4mm rostral to lesion, following injection of human recombinant NT-3 or cytochrome C (control). B. Sprouting at the lesion site, 1mm rostral to lesion, and 4mm rostral to lesion, following local injection of Ringer's solution (control), BDNF, NGF, or NT-3 in rats intracerebrally carrying hybridoma cells producing monoclonal antibody IN-1.

FIGURE 2. Millimeters of elongation of corticospinal tract fibers (mm from the lesion site) 14-17 days post-lesion in rats intracerebrally carrying hybridoma cells producing monoclonal antibody IN-1, following local injection of Ringer's (control), human recombinant BDNF, human recombinant NGF or human recombinant NT-3.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of promoting central nervous system regeneration in a subject in need of such treatment comprising administering a therapeutically effective amount of an

essentially purified and isolated neurotrophin family member together with an antibody directed toward a myelin-associated neurite growth inhibitory protein. In preferred, specific, non-limiting embodiments of the invention, the neurotrophin family member is NT-3 and the antibody directed toward a myelin-associated neurite growth inhibitory protein is IN-1 (which was raised to PAGE-purified 250,000d fraction from rat spinal cord myelin), as produced by hybridoma cell line IN-1 and deposited with the European Collection of Animal Cell Cultures (ECACC), PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, United Kingdom, and assigned accession number 88102801.

For purposes of clarity of description, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) isolation and purification of neurite growth regulatory factors;

(ii) protein characterization;

(iii) molecular cloning of genes or gene fragments encoding neurite growth regulatory factors;

(iv) production of antibodies to neurite growth regulatory factors; and

(v) methods of promoting central nervous system regeneration.

5.1. ISOLATION AND PURIFICATION OF NEURITE GROWTH REGULATORY FACTORS

The present invention relates to CNS myelin associated neurite growth inhibitory proteins. The CNS myelin associated inhibitory proteins of the invention may be isolated by first isolating myelin and subsequent purification therefrom. Isolation procedures which may be employed are described more

fully in the sections which follow. Alternatively, the CNS myelin associated inhibitory proteins may be obtained from a recombinant expression system (see Section 5.3., infra).

CNS myelin associated inhibitory proteins can be isolated from the CNS myelin of higher vertebrates including, but not limited to, birds or mammals. Myelin can be obtained from the optic nerve or from central nervous system tissue that includes but is not limited to spinal cords or brain stems. The tissue may be homogenized using procedures described in the art (Colman et al., 1982, J. Cell Biol. 95:598-608). The myelin fraction can be isolated subsequently also using procedures described (Colman et al., 1982, supra).

In one embodiment of the invention the CNS myelin associated inhibitory proteins can be solubilized in detergent (e.g., Nonidet P-40™, sodium deoxycholate). The solubilized proteins can subsequently be purified by various procedures known in the art, including but not limited to chromatography (e.g., ion exchange, affinity, and sizing chromatography), centrifugation, electrophoretic procedures, differential solubility, or by any other standard technique for the purification of proteins.

In specific embodiments, the NI-35 (35 Kd) and NI-250 (250 Kd) myelin-associated neurite growth inhibitory proteins may be utilized (Caroni and Schwab, 1988, J. Cell Biol. 106:1281-1288; Schwab and Caroni, 1988, J. Neurosci. 8:2381-2393; Caroni and Schwab, 1988, Neuron 1:85-96).

Alternatively, the CNS myelin associated inhibitory proteins may be isolated and purified using immunological procedures. For example, in one embodiment of the invention, the proteins can first be

solubilized using detergent (e.g., Nonidet P-40™, sodium deoxycholate). The proteins may then be isolated by immunoprecipitation with antibodies to the 35 kilodalton and/or the 250 kilodalton proteins. Alternatively, the CNS myelin associated inhibitory proteins may be isolated using immunoaffinity chromatography in which the proteins are applied to an antibody column in solubilized form.

5.2. PROTEIN CHARACTERIZATION

The neurite growth regulatory factors of the present invention can be characterized by assays based on their physical, immunological, or functional properties.

For example, the functional activity of a putative neurite growth inhibitory factor may be confirmed by testing the ability of the factor to inhibit sprouting or growth of neurites or spreading of 3T3 cells on a polylysine-coated tissue culture dish (Int. Application No. 899127864 filed November 2, 1989 by Schwab et al., U. S. Serial No. 07/401,212 by Schwab et al. filed August 30, 1989, and U. S. Serial No. 07/719,692 by Schwab et al. filed June 24, 1991). The half life of the neurite growth regulatory factors in cultured cells can be studied, for example, by use of cycloheximide, an inhibitor of protein synthesis (Vasquez, 1974, FEBS Lett. 40:563-584). In other experiments, a physiological receptor for a neurite growth regulatory factor could be identified by assays which detect complex formation with a neurite growth regulatory factor, e.g., by use of affinity chromatography with immobilized neurite growth regulatory factor, binding to a labeled neurite growth regulatory factor followed by cross-linking and immunoprecipitation, etc.

Electrophoretic techniques such as SDS-polyacrylamide gel electrophoresis and two-dimensional electrophoresis can be used to study protein structure. Other techniques which can be used include but are not limited to peptide mapping, isoelectric focusing, and chromatographic techniques.

The amino acid sequences of primary myelin associated inhibitors can be derived by deduction from the DNA sequence if such is available, or alternatively, by direct sequencing of the protein, e.g., with an automated amino acid sequencer. The protein sequences can be further characterized by a hydrophilicity analysis (Hopp and Woods, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the protein (and the corresponding regions of the gene sequence, if available, which encode such regions).

Secondary structural analysis (Chou and Fasman, 1974, Biochemistry 13:222) can also be done, to identify regions of the CNS myelin associated inhibitor sequence that assume specific secondary structures. Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstom, 1974, Biochem. Exp. Biol. 11:7-13) and computer modeling (Fletterick, R. and Zoller, M. (eds.), 1986, Computer Graphics and Molecular Modeling, in Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

5.3. MOLECULAR CLONING OF GENES OR GENE FRAGMENTS ENCODING NEURITE GROWTH REGULATORY FACTORS

5.3.1. ISOLATION AND CLONING OF THE NEURITE GROWTH REGULATORY FACTOR GENES

Any mammalian cell can potentially serve as the nucleic acid source for the molecular cloning of the genes encoding the CNS myelin associated inhibitory proteins, including but not limited to the 35 kD and/or 250 kD myelin associated proteins described in Caroni and Schwab (1988, Neuron 1:85-96).

The DNA may be obtained by standard procedures known in the art from cloned DNA (e.g., a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired mammalian cell. (See, for example, Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K., Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions, in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, a given neurite growth regulatory factor gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of a neurite growth regulatory factor gene from genomic DNA, DNA fragments are generated, some of which will encode the desired neurite growth regulatory factor gene. The DNA may be cleaved at specific sites using various restriction enzymes.

Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication.

The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing a neurite growth regulatory factor gene may be accomplished in a number of ways. For example, if an amount of a neurite growth regulatory factor gene or its specific RNA, or a fragment thereof, is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961-3965). For example, in a preferred embodiment, a portion of a neurite growth regulatory factor amino acid sequence can be used to deduce the DNA sequence, which DNA sequence can then be synthesized as an oligonucleotide for use as a hybridization probe.

Alternatively, if a purified neurite growth regulatory factor probe is unavailable, nucleic acid fractions enriched in neurite growth regulatory factor may be used as a probe, as an initial selection procedure.

It is also possible to identify an appropriate neurite growth regulatory factor-encoding fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further selection on the basis of the properties of the gene, or the physical, chemical, or immunological properties of its expressed product, as described supra, can be employed after the initial selection.

A neurite growth regulatory factor gene can also be identified by mRNA selection using nucleic acid hybridization followed by in vitro translation or translation in Xenopus oocytes. In an example of the latter procedure, oocytes are injected with total or size fractionated CNS mRNA populations, and the membrane-associated translation products are screened in a functional assay (3T3 cell spreading). Preadsorption of the RNA with complementary DNA (cDNA) pools leading to the absence of expressed inhibitory factors indicates the presence of the desired cDNA. Reduction of pool size will finally lead to isolation of a single cDNA clone. In an alternative procedure, DNA fragments can be used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified neurite growth regulatory factor DNA, or DNA that has been enriched for neurite growth regulatory factor sequences. Immunoprecipitation analysis or functional assays of the in vitro translation products of the isolated mRNAs identifies the mRNA and, therefore, the cDNA fragments that contain neurite growth regulatory factor sequences. An example of such a functional assay involves an assay for nonpermissiveness in which the effect of the various translation products on the spreading of 3T3 cells on a polylysine coated tissue culture dish is observed (see Caroni and Schwab, 1988, J. Cell Biol. 106:1281). In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against a neurite growth regulatory factor protein. A radiolabelled neurite growth regulatory factor cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe

to identify the neurite growth regulatory factor DNA fragments from among other genomic DNA fragments.

Alternatives to isolating the neurite growth regulatory factor genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the neurite growth regulatory factor gene. Other methods are possible and within the scope of the invention.

The identified and isolated gene or cDNA can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc.

In an alternative embodiment, the neurite growth regulatory factor gene may be identified and isolated after insertion into a suitable cloning vector, in a "shot gun" approach. Enrichment for a given neurite growth regulatory factor gene, for example, by size fractionation or subtraction of cDNA specific to low neurite growth regulatory factor producers, can be done before insertion into the cloning vector. In another embodiment, DNA may be inserted into an expression vector system, and the recombinant expression vector containing a neurite growth regulatory factor gene may then be detected by functional assays for the neurite growth regulatory factor protein.

The neurite growth regulatory factor gene is inserted into a cloning vector which can be used to transform, transfect, or infect appropriate host cells so that many copies of the gene sequences are generated. This can be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and neurite growth regulatory factor gene may be modified by homopolymeric tailing.

Identification of the cloned neurite growth regulatory factor gene can be accomplished in a number of ways based on the properties of the DNA itself, or alternatively, on the physical, immunological, or functional properties of its encoded protein. For example, the DNA itself may be detected by plaque or colony nucleic acid hybridization to labeled probes (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. and Hogness, D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Alternatively, the presence of a neurite growth regulatory factor gene may be detected by assays based on properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that inhibits <u>in vitro</u> neurite outgrowth. Further, a neurite growth regulatory factor protein may be identified by

detecting binding of antibody directed toward the factor to putative neurite growth regulatory factor-synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated neurite growth regulatory factor gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

If the ultimate goal is to insert the gene into virus expression vectors such as vaccinia virus or adenovirus, the recombinant DNA molecule that incorporates a neurite growth regulatory factor gene can be modified so that the gene is flanked by virus sequences that allow for genetic recombination in cells infected with the virus so that the gene can be inserted into the viral genome.

After the neurite growth regulatory factor DNA-containing clone has been identified, grown, and harvested, its DNA insert may be characterized as described in Section 5.3.4, infra. When the genetic structure of a neurite growth regulatory factor gene is known, it is possible to manipulate the structure for optimal use in the present invention. For example, promoter DNA may be ligated 5' of a neurite growth regulatory factor coding sequence, in addition to or replacement of the native promoter to provide for increased expression of the protein. Many manipulations are possible, and within the scope of the present invention.

5.3.2. EXPRESSION OF THE CLONED NEURITE GROWTH REGULATORY FACTOR GENES

The nucleotide sequence coding for a neurite growth regulatory factor protein or a portion thereof, can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcription and translation signals can also be supplied by the native neurite growth regulatory factor gene and/or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression elements of these vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/ translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination).

Expression vectors containing neurite growth regulatory factor gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions,

and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted neurite growth regulatory factor gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if a given neurite growth regulatory factor gene is inserted within the marker gene sequence of the vector, recombinants containing the neurite growth regulatory factor insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based on the physical, immunological, or functional properties of a given neurite growth regulatory factor gene product.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered neurite growth regulatory factor protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian (e.g. COS) cells can be used to ensure "native" glycosylation of the heterologous neurite growth regulatory factor protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extends.

5.3.3. IDENTIFICATION AND PURIFICATION OF THE EXPRESSED GENE PRODUCT

Once a recombinant which expresses a given neurite growth regulatory factor gene is identified, the gene product can be purified as described in Section 5.1, supra, and analyzed as described in Section 5.2, supra.

The amino acid sequence of a given neurite growth regulatory factor protein can be deduced from the nucleotide sequence of the cloned gene, allowing the protein, or a fragment thereof, to be synthesized by

standard chemical methods known in the art (e.g., see Hunkapiller, et al., 1984, Nature 310:105-111).

In particular embodiments of the present invention, such neurite growth regulatory factor proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods, include but are not limited to those containing altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are neurite growth regulatory factor proteins which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, etc.

5.3.4. CHARACTERIZATION OF THE NEURITE GROWTH REGULATORY FACTOR GENES

The structure of a given neurite growth regulatory factor gene can be analyzed by various methods known in the art.

The cloned DNA or CDNA corresponding to a given neurite growth regulatory factor gene can be analyzed by methods including but not limited to Southern hybridization (Southern, 1975, J. Mol. Biol. 98:503-517), Northern hybridization (Alwine, et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5350-5354; Wahl, et al., 1987, Meth. Enzymol. 152:572-581), restriction endonuclease mapping (Maniatis, et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), and DNA sequence analysis.

DNA sequence analysis can be performed by any techniques known in the art including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger, et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467), or use of an automated DNA sequenator (e.g., Applied Biosystems, Foster City, CA).

5.4. PRODUCTION OF ANTIBODIES TO NEURITE GROWTH REGULATORY FACTORS

Antibodies can be produced which recognize neurite growth regulatory factors or related proteins. Such antibodies can be polyclonal or monoclonal.

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a given neurite growth regulatory factor. For the production of antibody, various host animals can be immunized by injection with a neurite growth regulatory factor protein, or a synthetic protein, or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such

as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

A monoclonal antibody to an epitope of a neurite growth regulatory factor can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

In preferred embodiments of the invention, the monoclonal antibody is produced by cell line IN-1, deposited with ECACC and having accession number 88102801. In additional embodiments, the monoclonal antibody is produced by cell line IN-2, deposited with the ECACC and having accession number 88102802.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

A molecular clone of an antibody to a neurite growth regulatory factor epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragment's include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the 2 Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

5.5. METHODS OF PROMOTING CENTRAL NERVOUS SYSTEM REGENERATION

The present invention relates to methods of promoting central nervous system regeneration in a subject in need of such treatment comprising administering a therapeutically effective amount of an essentially purified and isolated neurotrophin family member together with an antibody directed toward a myelin-associated neurite growth inhibitory protein.

Neurotrophin family members include, but are not limited to, BDNF, as described in PCT Publication No. WO 91/03568 published March 21, 1991 (corresponding to

United States Serial No. 07/570,657 by Barde et al.); NT-3, as described in PCT publication No. WO 91/03569 published March 21, 1991 (corresponding to United States Serial No. 07/570,189 by Barde et al.); NGF, as described in United States Patent No. 5,169,762 by Gray et al., issued December 8, 1992; and NT-4, as described in PCT publication No. WO 92/20365. Preferably, the species of origin of neurotrophin used is the same species as the subject being treated. The neurotrophin may be essentially purified and isolated using methods set forth in the cited references or known in the art.

Antibodies that may be used according to the invention include, but are not limited to, IN-1.

Methods of promoting central nervous system regeneration may be measured by quantitatively or qualitatively evaluating neurite sprouting or fiber extension or by evaluating recovery of neurological function, using clinical parameters or methods such as those set forth in Section 6, infra.

Subjects in need of such treatment include human as well as non-human subjects suffering from a disorder of the central nervous system including but not limited to a disorder caused by trauma, infarction, infection, embolism, malignancy, metabolic defect, exposure to a toxin, degenerative disorder, etc. In a preferred, nonlimiting embodiment of the invention, the subject is a human suffering from a neurological disorder that involves the corticospinal tract, including, but not limited to, spinal cord trauma, amyotrophic lateral sclerosis, primary lateral sclerosis, ischemia, stroke, multiple sclerosis, compression lesions, syringomyelia, and multiple systems degeneration. In another preferred, nonlimiting embodiment of the invention, the subject

is a human suffering from a neurological disorder that involves the optic nerve.

The term "treatment", as used herein, refers to the amelioration of symptoms associated with the neurological disorder or a prolongation of survival. In certain instances, a "cure" may be achieved, but the present invention is not so limited.

A therapeutically effective amount of neurotrophin and antibody refers to that amount that results in amelioration of symptoms or a prolongation of survival in a subject in need of such treatment. In various embodiments of the invention, the local concentration of neurotrophin may be between about 0.01 and 100 nanograms per gram tissue (net weight) and the local concentration of antibody directed toward myelin-associated neurite growth inhibitory protein may be between about 0.01 and 10 micrograms per gram tissue. Dosage may be determined using standard techniques, e.g. as described in Fingl and Woodbury, 1975, in "The Pharmacological Basis of Therapeutics," Fifth Edition, Goodman and Gilman, eds., Macmillin Publ., N. Y., pp. 1-46.

Neurotrophin and antibody may be administered by any suitable route, including, but not limited to, local application via surgery or injection, intravenous, intrathecal, subcutaneous, or intramuscular routes. Neurotrophin and antibody may also be administered via a cellular implant that secretes neurotrophin or antibody. Neurotrophin and antibody may be administered either together or separately by different routes. It is preferred, however, that concurrent exposure to both neurotrophin and antibody be achieved.

The present invention also provides for pharmaceutical compositions comprising neurotrophin

and antibody directed toward myelin-associated neurite growth inhibitory protein in a suitable pharmaceutical carrier.

6. EXAMPLE: NEUROTROPHIN 3 (NT-3) ENHANCES REGENERATIVE SPROUTING OF THE LESIONED CORTICOSPINAL TRACT

6.1. MATERIALS AND METHODS

The spinal cord of young adult, 4-7 week old rats was lesioned at the mid-thoracic level by bilateral transection of the dorsal half with iridectomy scissors. Recombinant human neurotrophic factors NGF, BDNF, or NT-3 (300 - 500 ng in 0.3-0.5 μl) were injected with a glass capillary immediately rostral to the lesion into the spinal cord. Controls received equivalent amounts of cytochrome C or vehicle (Ringer's solution). In some of the rats hybridoma cells producing the monoclonal antibody (mAB)IN-1 were intracerebrally injected into the left frontal cortex and lateral ventricle. This antibody was raised against the PAGE-purified 250,000 protein fraction from rat spinal cord myelin, a preparation which was highly enriched in neurite growth inhibitory activity. The antibodies secreted by the locally formed tumor reached the spinal cord via the cerebrospinal fluid. 14-17 days later, the corticospinal tract (CST) axons were traced by anterograde transport of wheat germ agglutinin-horseradish peroxidase (WGA-HRP) injected into the right sensory motor cortex. Rats were fixed and processed for HRP histochemistry one day later.

6.2. RESULTS

Sprouting was quantified on complete serial, parasagital sections by counting all the labelled branches intersecting vertical lines at the lesion

site 1 mm rostral and 4 mm rostral to the lesion. The numbers obtained were related to the number of labelled axons within the compact CST; the value for the normal collateral branching present in unlesioned animals was substracted in order to get a sprouting index.

Fig. 1a shows that spontaneous sprouting of lesioned adult CST fibers occurred at all three levels. A single injection of NT-3 at the time of lesion greatly increased this sprouting. The effect was visible at the lesion site and at 1 mm, but was decreased at 4 mm, perhaps due to a penetration of the factor (Fig. 1a). Control injections with cytochrome C (Fig. 1a) or Ringer's solution alone (Fig. 1b) were indistinguishable.

Comparison of three members of the neurotrophin family showed very significant differences (Fig. 1b). Whereas BDNF had little effect, sprouting seemed to be enhanced in the NGF-treated rats without, however, reaching significant levels (except at 4 mm). In contrast, a highly significant enhancement of sprouting was seen with NT-3. The number of labelled axons in the CST varied from animal to animal. However, the values for all the groups overlapped and the mean numbers for each treatment group were very similar (70-125 labelled axons per CST). This result shows that the neuronal metabolism, as shown by anterograde transport of the lectin coupled HRP (probably reflecting newly synthesized glycoproteins and glycolipids), was not affected by the trophic factor treatment.

The elongation of CST axons was determined on the same serial parasagittal sections for all the groups on number coded slides. The distance of the most caudal labelled fibers was measured (mm from the

lesion site) and is shown in Fig. 2. Regeneration distances of 0.2-0.7 mm correspond to the mean length of sprouts growing towards and around the lesion site. In presence of NT-3, i.e. under conditions of strongly stimulated sprouting, no elongation greater than 0.7 mm was observed in the presence of a control antibody. In contrast, 7 of 16 rats showed elongation over much longer distances with the combined treatment of NT-3 and mAB IN-1. In fact, this group contained animals showing the longest regenerations we have ever observed. In these rats, the regenerating fibers reached the lower lumbar and sacral spinal cord. Regeneration over distances of several millimeters were also obtained by combinations of mAB IN-1 with BDNF or NGF (Fig. 2).

6.3. DISCUSSION

The foregoing data shows that the local injection of neurotrophin 3 (NT-3) into the partially transacted spinal cord of adult rats increases the regenerative sprouting of the largest descending fiber tract, the corticospinal tract (CST). Among the related NGF family members, brain-derived neurotrophic factor (BDNF) had little effect, whereas NGF showed an intermediate effect on sprouting. In spite of this stimulation, only short regeneration distances (about 1 mm) from the lesion site were observed. In contrast, the application of a monoclonal antibody (IN-1) raised against the myelin-associated neurite growth inhibitors alone or in combination with neurotrophic factors resulted in long distance regeneration (2-20 mm) of a small percentage of CST fibers. Enhancement of the spontaneous regeneration attempts of CNS neurons by specific neurotrophic factors and counteraction of the inhibitory substrate effects of

adult CNS tissue may thus cooperate to improve regeneration of lesioned nerve fiber tracts in the CNS.

Various references are cited herein which are hereby incorporated by reference in their entirety.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 21, line 23-24

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

European Collection of Animal Cell Cultures (ECACC)

Address of depositary institution *(including postal code and country)*

Vaccine Research & Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research
Porton Down, Salisbury, Wiltshire
United Kingdom SP4 0JG

Date of deposit	Accession Number
28.10.1988	88102802

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer: A. Loris	Authorized officer

Form PCT/RO/134 (July 1992)

WHAT IS CLAIMED IS:

1. A method of promoting central nervous system regeneration in a subject in need of such treatment comprising administering to the subject a therapeutically effective amount of a purified and isolated neurotrophin family member together with an antibody that specifically binds a myelin-associated neurite growth inhibitory protein.

2. The method of claim 1 in which the subject in need of such treatment suffers from a neurological disorder involving the corticospinal tract.

3. The method of claim 2 in which the neurological disorder is a degenerative condition.

4. The method of claim 1 in which the subject in need of such treatment suffers from a neurological disorder that is a degenerative condition.

5. The method of claim 3 in which the neurological disorder is amyotrophic lateral sclerosis.

6. The method of claim 1, 2, 3, 4, or 5 in which the neurotrophin family member is neurotrophin-3.

7. The method of claim 1, 2, 3, 4, or 5 in which the antibody is monoclonal antibody IN-1, as produced by cell line IN-1, deposited with the European Collection of Animal Cell Cultures and assigned accession number 88102801.

8. The method of claim 1, 2, 3, 4, or 5 in which the neurotrophin family member is neurotrophin-3 and the antibody is monoclonal antibody IN-1, as produced by cell line IN-1, deposited with the European Collection of Animal Cell Cultures and assigned accession number 88102801.

9. A pharmaceutical composition comprising a purified and isolated neurotrophin family member and an antibody that specifically binds a myelin-associated neurite growth inhibitory protein in a suitable pharmaceutical carrier.

10. The pharmaceutical composition of claim 9 in which the neurotrophin family member is neurotrophin-3.

11. The pharmaceutical composition of claim 9 in which the antibody is monoclonal antibody IN-1, as produced by cell line IN-1, deposited with the European Collection of Animal Cell Cultures and assigned accession number 88102801.

12. The pharmaceutical composition of claim 9 in which the neurotrophin family member is neurotrophin-3 and the antibody is monoclonal antibody IN-1, as produced by cell line IN-1, deposited with the European Collection of Animal Cell Cultures and assigned accession number 88102801.

13. A method of promoting corticospinal tract regeneration in a subject in need of such treatment comprising administering to the subject a therapeutically effective amount of purified and isolated neurotrophin-3 together with monoclonal

antibody IN-1, as produced by cell line IN-1, deposited with the European Collection of Animal Cell Cultures and assigned accession number 88102801.

14. The method of claim 13 in which the subject suffers from amyotrophic lateral sclerosis.

15. The method of claim 1 in which the central nervous system regeneration is optic nerve regeneration.

16. The method of claim 15 in which the neurotrophin family member is brain-derived neurotrophic factor.

17. The method of claim 16 in which the antibody is monoclonal antibody IN-1, as produced by cell line IN-1, deposited with the European Collection of Animal Cell Cultures and assigned accession number 88102801.

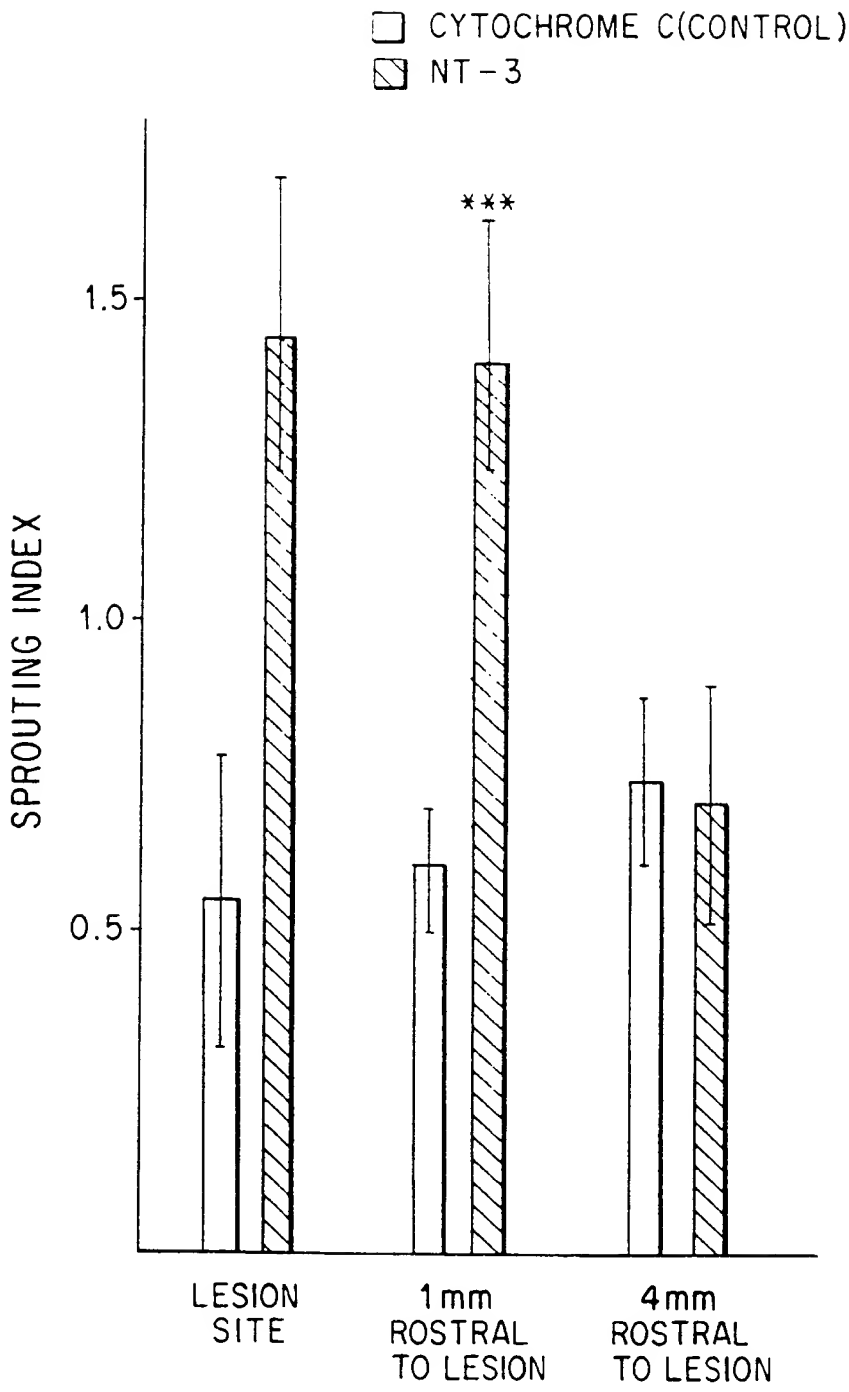
CYTOCHROME C(CONTROL)
NT-3
SPROUTING INDEX
1.5
1.0
0.5
***
LESION SITE
1mm ROSTRAL TO LESION
4mm ROSTRAL TO LESION

FIG. 1A

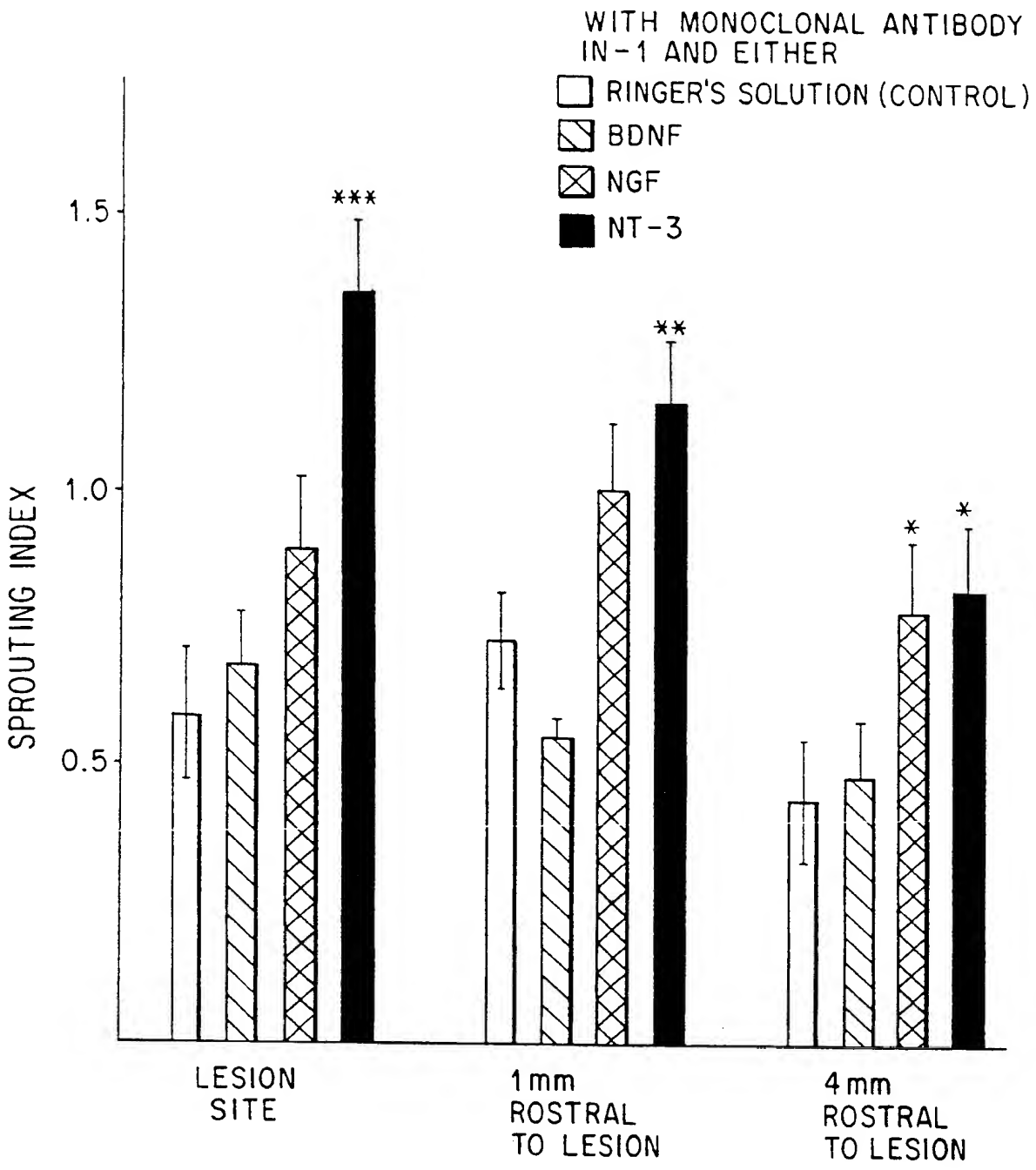
WITH MONOCLONAL ANTIBODY IN-1 AND EITHER
RINGER'S SOLUTION (CONTROL)
BDNF
NGF
NT-3
SPROUTING INDEX
1.5
1.0
0.5
***
**
*
*
LESION SITE
1 mm ROSTRAL TO LESION
4 mm ROSTRAL TO LESION

FIG. 1B

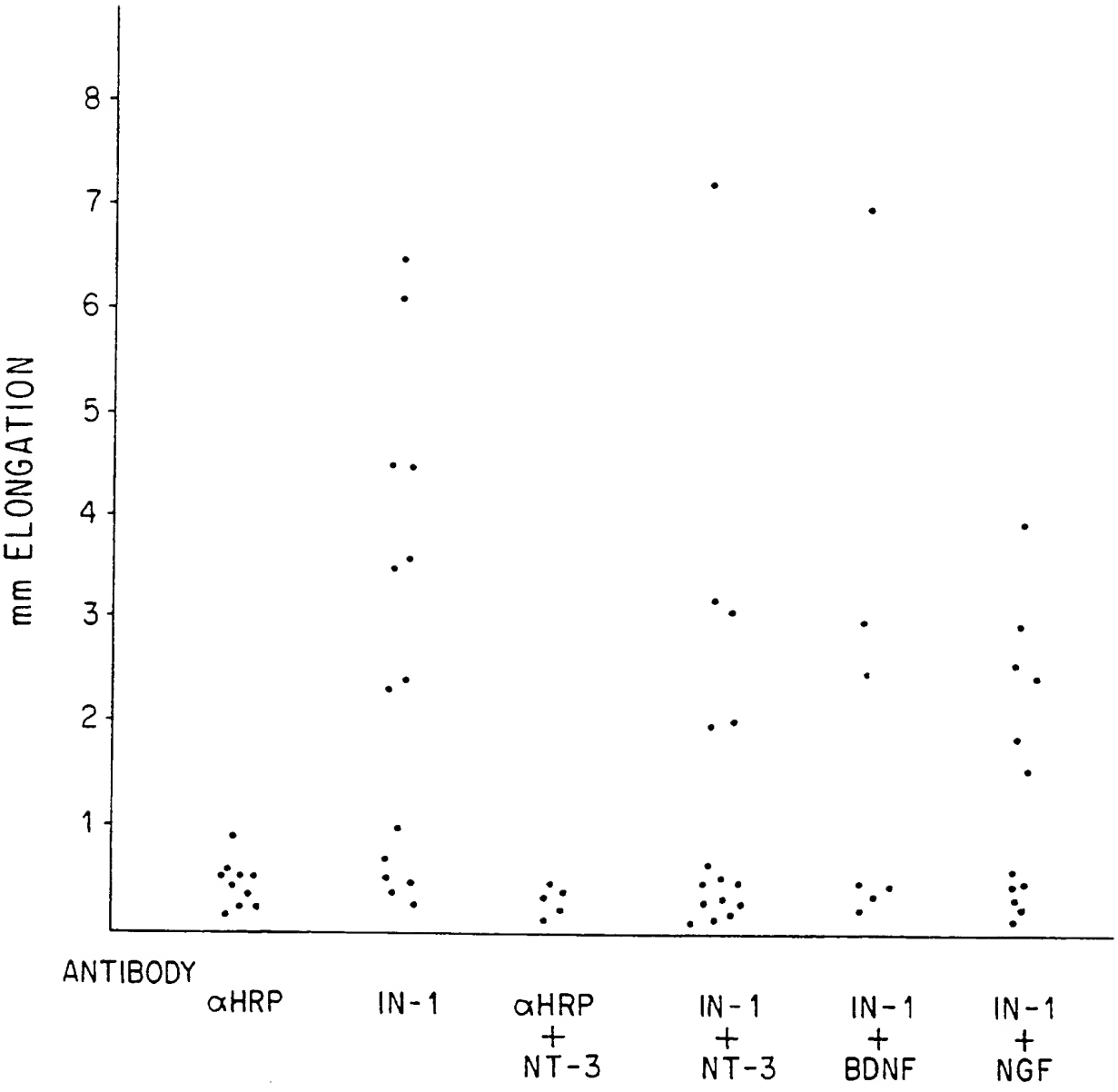
mm ELONGATION
8
7
6
5
4
3
2
1
ANTIBODY
αHRP
IN-1
αHRP + NT-3
IN-1 + NT-3
IN-1 + BDNF
IN-1 + NGF

FIG. 2

INTERNATIONAL SEARCH REPORT

Interr tal Application No
PCT/IB 94/00011

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NATURE (UNITED KINGDOM), 1994, VOL. 367, NO. 6459, PAGE(S) 170-173, Schnell L. et al 'Neurotrophin -3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion' see the whole document ---	1-17
P,X	HOST: BRS DATABASE: CRSZ AN 4307755. 9312. PROJECT NUMBER: K08NS01602-01A1. TI NEUROTROPHIC FACTORS AND CNS TRANSPLANTATION. AU HELM, G.A., UNIVERSITY OF VIRGINIA, US see entire on-line abstract --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 June 1994	16.06.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Dullaart, A

Form PCT/ISA/210 (second sheet) (July 1992)

Intern al Application No
PCT/IB 94/00011

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,90 05191 (ERZIEHUNGSDIREKTION KANTON ZURICH (ERZI-) ERZIEHUNGSDIREKTION) 17 May 1990 see abstract see page 5 see page 9 see paragraph 5.4 see page 73, line 10 - page 74, line 23 see paragraph 8; claims 1,2,24-35	1-17
Y	WO,A,93 00427 (ERZIEHUNGSDIREKTION CANTON ZURICH) 7 January 1993 see page 1 see page 11 see paragraph 5.4 see page 87	1-17
Y	WO,A,91 03569 (MAX PLANCK GES FOERDERUNG WISSENSCHAFTEN (REGE-) REGENERON PHA) 21 March 1991 see abstract see page 12 - page 13 see paragraph 5.4 see paragraph 5.7 -paragraph 5.7.2 see paragraph 9; claims 82-98	1-17
P,Y	DATABASE WPI Section Ch, Week 9318, Derwent Publications Ltd., London, GB; Class B04, AN 93-152039 & US,A,7 890 713 (KAPLAN D ET AL) 1 April 1993 see abstract	1-17

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 94/00011

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
REMARK: Although claims 1-8 and 13-17 are directed to a method of treatment of the human/animal body (PCT Rule 39.1(IV)), the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1-11, 13-17
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
In view of the large number of compounds, which are defined by the general definition of the active compounds, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds .....(please see annex)

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

....mentioned inthe claims, and to the general idea underlying the application (see guidelines, Part B, Chapter III, paragraph 3.6).

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna[illegible]al Application No
PCT/IB 94/00011

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9005191	17-05-90	AU-A-	4528089	28-05-90
		EP-A-	0396719	14-11-90
		JP-A-	4117298	17-04-92
		US-A-	5250414	05-10-93
WO-A-9300427	07-01-93	US-A-	5250414	05-10-93
		AU-A-	2152092	25-01-93
WO-A-9103569	21-03-91	US-A-	5180820	19-01-93
		AU-B-	647412	24-03-94
		AU-A-	6337390	08-04-91
		AU-B-	643705	25-11-93
		AU-A-	6404990	08-04-91
		CA-A-	2040412	01-03-91
		CN-A-	1052141	12-06-91
		CN-A-	1052142	12-06-91
		EP-A-	0440777	14-08-91
		EP-A-	0441947	21-08-91
		JP-A-	5161493	29-06-93
		PT-B-	95153	31-12-93
		WO-A-	9103568	21-03-91
		US-A-	5229500	20-07-93